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Newly Identified Vitamin K-producing Bacteria Isolated from the Neonatal Faecal Flora

Gordon Cooke

Technological University Dublin, gordon.cooke@tudublin.ie

John Behan

Technological University Dublin, john.behan@tudublin.ie

Mary Costello

Technological University Dublin, mary.costello@tudublin.ie

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Newly identified Vitamin K producing bacteria originating in the neonatal faecal flora

Gordon B. Cooke, John F. Behan and Mary C. Costello *

Department Science, ITT Dublin, Tallaght, Dublin 24, Ireland

*Correspondence author:

Mary Costello mary.costello@ittdublin.ie

Phone: +353 (0)1 4042 806

Fax: +353 (0)1 4042 700

Abstract

Fat soluble vitamin K is an essential component of the blood clotting process. Menaquinones are the naturally occurring form of vitamin K produced by bacteria. Lipid extracts were made from three bacteria originally isolated from the human neonatal gut and identified as *Enterobacter agglomerans*, *Serratia marcescens* and *Enterococcus faecium*. Following preparative TLC the lipid extracts were subjected to LC-MS analysis. Peak analysis of the LC MS data showed that the three bacteria produce various forms of menaquinone.

Key words: Vitamin K, Menaquinone, *Enterobacter agglomerans*, *Serratia marcescens*, *Enterococcus faecium*, Neonatal gut microflora

1. Introduction

Vitamin K is a generic term for a family of fat soluble vitamins. Two of the naturally occurring quinine forms are: phyloquinone (vitamin K₁ or 2-methyl-3-eicosa-2'-ene-1,4-naphthoquinone) which is obtained from plants and menaquinone (vitamin K₂ or 2-methyl-3-multiprenyl-1,4-naphthoquinone), which is produced by certain bacteria during anaerobic respiration(1,2) Menaquinone variants are differentiated based on the number of isoprene-5-carbon prenyl units on their side chains. Chain length varies from 2-15 isoprene units (3). The variations in the structure of the menaquinones produced by bacteria have been used in bacterial classification (4). Several bacteria that have been isolated from the human intestine have previously been identified as producing menaquinones of various chain lengths and it is thought that these bacteria contribute to the vitamin K requirements of the human body (5). This paper discusses the identification of three previously unreported menaquinone producing bacteria isolated from faecal samples of neonates.

2. Materials and Methods

2.1 Cultures and Cultivation

Cultures were isolated from faecal samples obtained from neonates aged between birth and 6 weeks old in a study previously carried out within our laboratory (6). The isolates were identified using selective media, Gram stains and API tests. Purified cultures were grown in 1 litre Durans containing tryptone soy broth powder (Lab M) dissolved in deionised water with haemin (10mg) and cysteine (0.05%w/v) (3). The cultures were incubated anaerobically at 37°C for 72 hours. Cultures were checked for purity using Grams stains, selective and non-selective agar. The bacterial pellets were harvested in a Sorvall RC-5B centrifuge at 16,266g for 10 minutes at 4°C. The pellet was washed 3 times in 20ml volumes of 0.01M sterile phosphate buffered saline (PBS) and centrifuged again under the same conditions. Menaquinone 4 standard was purchased from Sigma, UK.

2.2 Extraction, Purification and Analysis of Menaquinones

Extraction of suspect menaquinones from the wet bacterial pellets was carried out using the modified Bligh and Dyer method (7) developed by Hammond et al. (8), which involves the extraction of total lipid content. Menaquinone 4, currently the only commercially available menaquinone, was used as a standard as menaquinones of different side chain lengths migrate similar distances on thin layer chromatographic (TLC) plates (9). The

extracted menaquinones were then purified using the procedure as described by Fernandez et al. (9), on preparative silica gel GF₂₅₄ prep TLC plates. The suspect menaquinones were viewed under a UV light box emitting light at 254nm. Bands from the lipid extracts of the bacteria that were in line with the menaquinone 4 standard were removed and dissolved in dichloromethane:methanol::30:70, as was the menaquinone 4 standard, and all were subsequently analysed by mass spectrometry. Liquid chromatography-Mass spectrometry (LC-MS) analysis was carried out using a Bruker Daltronics Esquire liquid chromatography ion trap mass spectrometer with a Synergi Hydro C18 reverse phase (4.6mm X 250mm) HPLC column (00G-4375-E0) supplied by Phenomenex of Cheshire, UK using the conditions described in Table 1.

3. Results

Previous studies have shown that menaquinones of differing side chain length give similar R_f values following TLC analysis (9) Therefore lipids extracts with bands which co-migrated with the menaquinone 4 standard (unpublished results) were subjected to further analysis to determine the exact menaquinone form produced by the respective bacteria being studied.

The LC-MS data obtained for the standard menaquinone 4 are presented in Figure 1. Peak X, indicated in the total ion count shown in Figure 1(a) was further analysed yielding the three significant peaks shown in Figure 1(b). The molecular weights of the three peaks in Figure 1(b) correlated to the molecular weights of the protonated (mass = 445.3), sodiated (mass = 467.4) and potassiated (mass = 483.1) forms of menaquinone 4.

LC-MS analysis of the extract from *Enterobacter agglomerans* (Figure 2a – peak A) showed molecular weight bands at 717.5 (protonated), 739.7 (sodiated) and 755.5 (potassiated) (Figure 2b) correlating to the expected molecular weight for Menaquinone 8 analogues. The sodiated analogue of Menaquinone 8 forms the base peak analogue as was detected for the Menaquinone 4 standard (Figure 1b).

In addition to *E.agglomerans*, an isolate of *Serratia marcescens* was also found to produce menaquinone 8 (Fig. 3a). As with the analysis of

E. agglomerans, the mass spectrometric analysis showed molecular weights corresponding to the protonated, sodiated and potassiated forms and the base peak as with the other bacterial extracts was the sodiated form of menaquinone 8 (Figure 3b).

The peak detected for *Enterococcus faecium* TLC lipid extract (Figure 4a – Peak C), although of weak intensity, gave sufficient signal to allow for identification as menaquinone 7. As with the analysis for the previous strains, the protonated, sodiated and potassiated forms of menaquinone were detected, with the sodiated analogue forming the base peak.

4. Discussion

The LC-MS results presented in this paper for the three bacterial strains *E. agglomerans*, *S. marcescens* and *E. faecium* show peaks correlating with sodiated, protonated and hydrogenated derivatives of the menaquinones. Molecules of sodium, potassium and hydrogen are abundant in the environment and in this experiment they could be sourced from the glass vials used to store the samples. Ion contaminants have been reported previously to form apparently stable complexes with the menaquinones during electro-spray ionisation (10). The sodium, which forms the base peak analogue, is most likely forming weak covalent bonds with the oxygen on the ring structures of the menaquinones, as are the hydrogen and potassium.

This previously unreported identification of the strains *E.agglomerans*, *S.marcescens* and *E.faecium* as menaquinone producers will in the first instance contribute to aiding in their microbiological classification (11). In addition, the sourcing of these additional vitamin K producing strains from the neonatal gut flora show their potential as bacteria which could contribute in the neonate to the overall requirements of the vitamin K, an essential vitamin in the human blood clotting process (1). In the study presented here, the newly identified vitamin K producing strains together with several other previously reported vitamin K producing strains (*Bacteroides ovatus*, *Citrobacter freundii*, *Enterococcus faecalis*, *Escherichia coli*, *Prevotella buccae*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus warneri*) were all isolated in infants of less than six weeks of age (unpublished results). Further studies would be required to confirm if the bacteria listed above are contributing significantly to the overall vitamin K requirement of the newborn infant at this age which could have implications in relation to the duration of hospital regimes for oral administration of vitamin K (12,13) as a prophylactic treatment for the prevention of haemorrhagic disease of the newborn (14).

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Table 1. Conditions Used for Monitoring of Menaquinones on the Bruker Esquire-LC Ion Trap LC/MS.

HPLC System	Bruker Esquire-LC Ion Trap LC/MS
Column	Synergi Hydro Reverse Phase C18 Column (250x4.6mm)
Detector	Ion Trap
Mobile Phase	Dichloromethane:Methanol (30:70)
Monitored Wavelength	270nm
Flow Rate	0.3 and 0.4mls/min
Sample Size Loaded	Various
Scan Begin	50.00 m/z
Scan End	2200.00 m/z
Ion Polarity	Positive
Ion Source Type	Electro Spray Ionisation
Sample Run Time	45-60 minutes

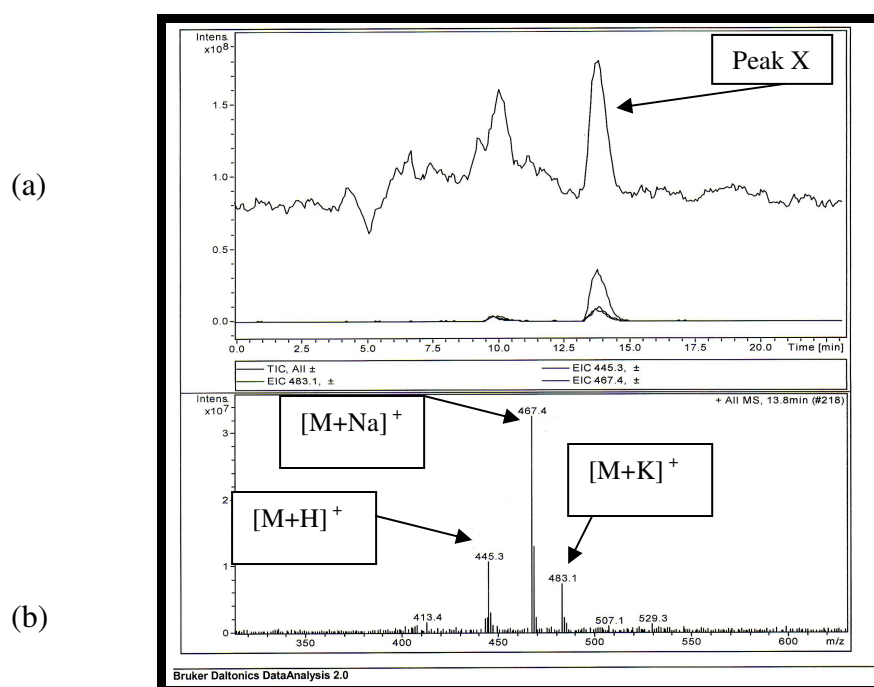


Figure 1 Mass spectrometric analysis of menaquinone standard using the conditions as listed in Table 1.

(a) Total ion count of a directly injected menaquinone 4 standard (6 μ g/ml).

(b) Mass spectrometric analysis of peak X (figure 1a) detected at 13.8 minutes.

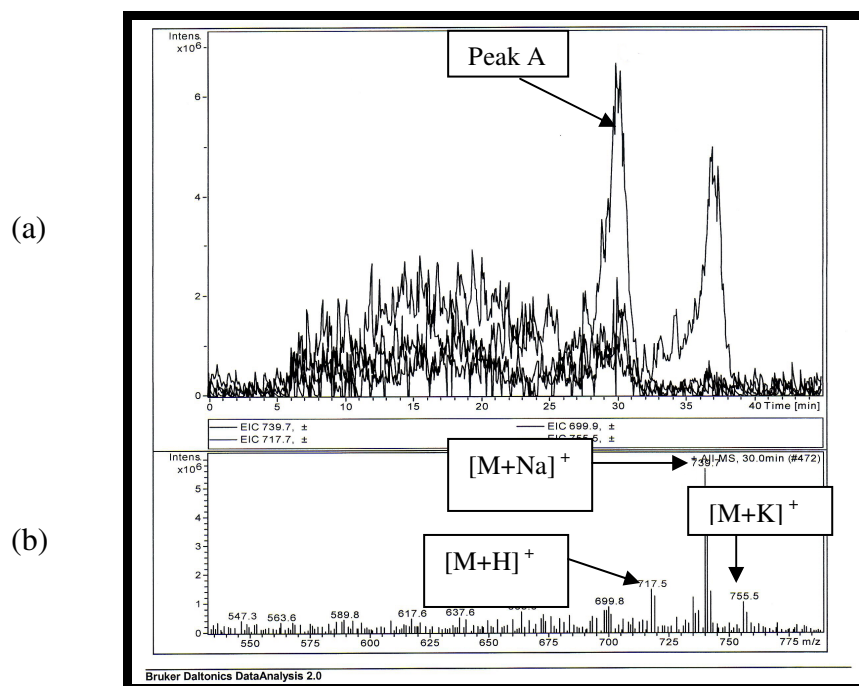


Figure 2 Mass spectrometric analysis of *Enterobacter agglomerans* extract carried out under the conditions listed in Table 1

(a) Extrapolated ion count for a TLC extract prepared from an *Enterobacter agglomerans* extract.

(b) Mass spectrometric analysis of peak A detected at a retention time of 30 minutes.

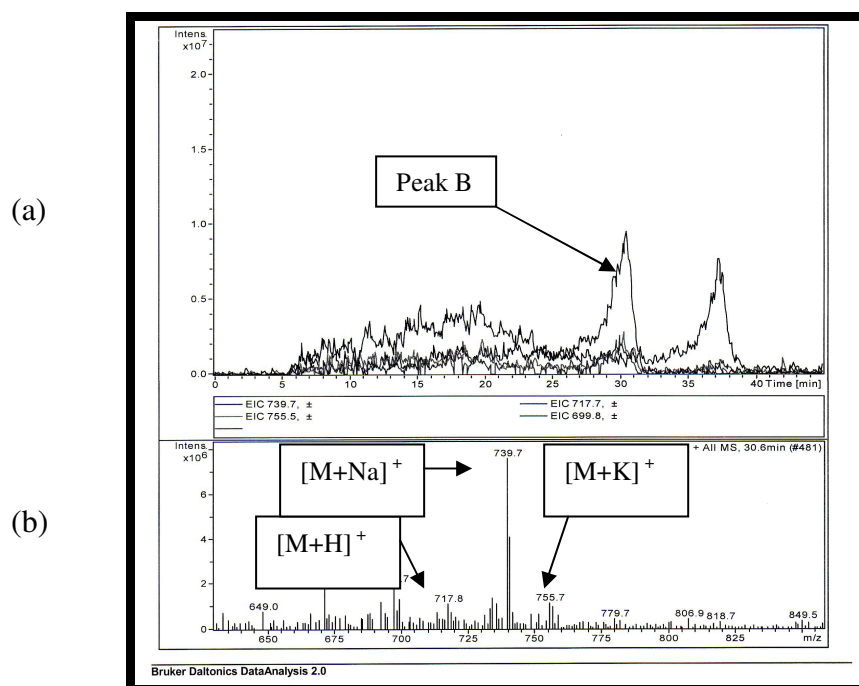


Figure 3 Mass spectrometric analysis of a *Serratia marcescens* extract carried out using the conditions as described in Table 1.

(a) Extrapolated ion count for a TLC extract of *Serratia marcescens* extract.

(b) Mass spectrometric analysis of peak B detected at a retention time of 30.6 minutes.

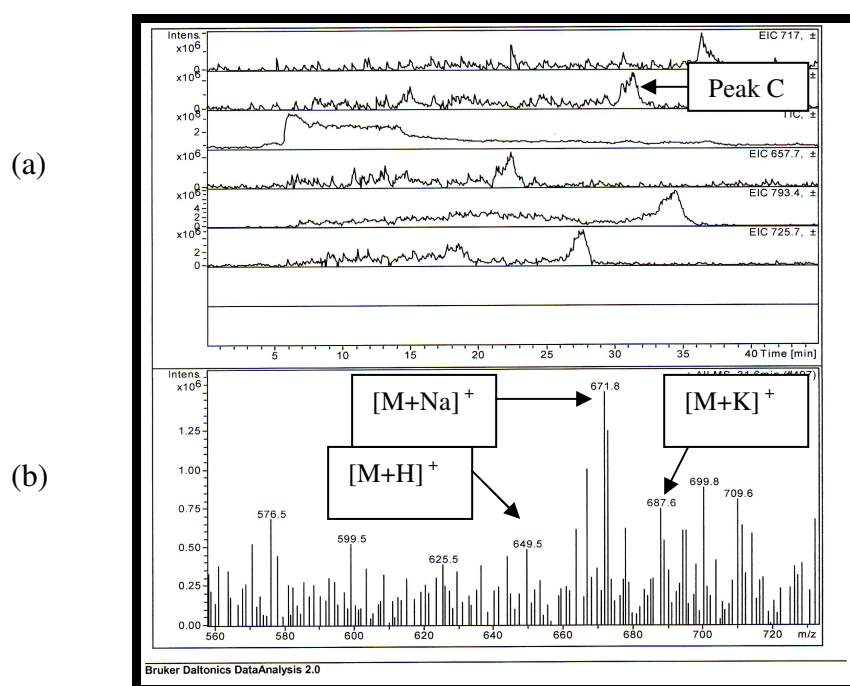


Figure 4 Mass spectrometric analysis of a *Enterococcus faecium* TLC extract analysed using the conditions as listed in Table 1.

(a) Extrapolated ion count for individual peaks and total ion counts for a TLC extract obtained from an *Enterococcus faecium*.

(b) Mass spectrometric analysis of the Peak C detected at a retention time of 31.6 minutes.